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Pathology of *Lagovirus europaeus* GI.2/RHDV2/b (Rabbit Hemorrhagic Disease Virus 2) in Native North American Lagomorphs

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ABSTRACT: Rabbit hemorrhagic disease, a notifiable foreign animal disease in the US, was reported for the first time in wild native North American lagomorphs in April 2020 in the southwestern US. Affected species included the desert cottontail (*Sylvilagus audubonii*), mountain cottontail (*Sylvilagus nuttallii*), black-tailed jackrabbit (*Lepus californicus*), and antelope jackrabbit (*Lepus alleni*). Desert cottontails (n=7) and black-tailed jackrabbits (n=7) collected in April and May 2020 were necropsied at the US Geological Survey National Wildlife Health Center and tested positive for *Lagovirus europaeus* GI.2, also known as rabbit hemorrhagic disease virus 2 (GI.2/RHDV2/b), by real-time PCR at the US Department of Agriculture's Foreign Animal Disease Diagnostic Laboratory. Gross and microscopic lesions were similar to those reported in European rabbits (*Oryctolagus cuniculus*) and other hare (*Lepus*) species with GI.2/RHDV2/b infection; they included epistaxis (12/13; 92%); massive hepatocellular dissociation (14/14; 100%) and necrosis or apoptosis (11/11; 100%); pulmonary congestion (12/12; 100%), edema (12/13; 92%), and hemorrhage (11/12; 92%); and acute renal tubular injury (3/8; 38%). As in previous reports, massive hepatocellular dissociation and necrosis or apoptosis was the most diagnostically distinct finding. As North American *Sylvilagus* and *Lepus* species appear to be susceptible to fatal GI.2/RHDV2/b infection, additional work is needed to understand the host range, pathogenicity, and potential population effects of GI.2/RHDV2/b in North America.

Key words: Cottontail, jackrabbit, *Lagovirus*, *Lepus* spp., pathology, rabbit hemorrhagic disease virus 2, *Sylvilagus* spp.

Rabbit hemorrhagic disease virus (*Lagovirus europaeus* GI.1/RHDVa) is a *Lagovirus* in the family *Caliciviridae*, with a host range primarily limited to the European rabbit (*Oryctolagus cuniculus*; Abrantes et al. 2012). Following detection in China in 1984, the virus quickly spread through Asia, Europe, and Australia, causing heavy losses of commercial and wild *O. cuniculus* (Abrantes et al. 2012). A second pathogenic *Lagovirus*, rabbit hemorrhagic disease virus 2 (*Lagovirus europaeus* GI.2/RHDV2/b), emerged in France in 2010 (Le Gall-Reculé et al. 2013). This strain also exhibits high virulence in *O. cuniculus* and has spread rapidly throughout the world, leading to additional economic and ecologic disruptions due to its effect on domestic and wild rabbit populations (Rouco et al. 2019). Compared to GI.1/RHDVa, GI.2/RHDV2/b shows: a broader host range, including multiple hare species (e.g., brown hare [*Lepus europaeus*; Velarde et al. 2017] and mountain hare [*Lepus timidus*; Neimanis et al. 2018a]); a lower but highly variable mortality rate; the potential for longer disease duration; and the ability to cause lethal infection in young animals as well as adults (Velarde et al. 2017).

The first detection of GI.2/RHDV2/b in North America occurred in 2016 in domestic *O. cuniculus*, with limited, sporadic detections in subsequent years (Rouco et al. 2019). In April 2020, GI.2/RHDV2/b was reported in native North American lagomorphs associated with mortality events involving wild desert cottontail (*Sylvilagus audubonii*) and black-tailed jackrabbit (*Lepus californicus*) in New Mexico, US (World Organization for Animal Health 2020). In the following months, mortalities of multiple wild rabbit and hare species, including desert cottontail, mountain cottontail (*Sylvilagus nuttallii*), black-tailed jackrabbit, and antelope jackrabbit (*Lepus alleni*), were reported in Arizona, California, Colorado, Nevada, Texas, and Utah, in association with GI.2/RHDV2/b detection (US Geological Survey National Wildlife Health Center 2020). As rabbit hemorrhagic disease was unprecedented in native North

American wildlife, little was known regarding either the pathology or virulence of GI.2/RHDV2/b in these species. Here, we report postmortem findings in desert cottontails and black-tailed jackrabbits naturally infected with GI.2/RHDV2/b and compare them to findings in Old World lagomorph species.

Seven desert cottontails and seven black-tailed jackrabbits from Texas (n=9), New Mexico (n=4), and Arizona (n=1), collected in April and May 2020, were received for postmortem examination at the US Geological Survey National Wildlife Health Center. Animals were necropsied, and liver, lung, kidney, spleen, brain, trachea, heart, eye, esophagus, stomach, duodenum, jejunum, cecum, and colon were collected in 10% neutral buffered formalin, processed routinely, sectioned at approximately 5 μm, and stained with H&E for light microscopic assessment (not all tissues were examined from all animals, depending on the degree of postmortem artifact). A subset of liver, kidney, and lung sections was stained with phosphotungstic acid–hematoxylin to highlight fibrin, while another subset of liver sections was stained with von Kossa to highlight calcification. Liver was tested for GI.1/RHDVa and GI.2/RHDV2/b by real-time-PCR at the US Department of Agriculture's Foreign Animal Disease Diagnostic Laboratory, Plum Island, New York (FADDL) (adapted from Duarte et al. 2015; US Department of Agriculture 2018). Age assessment was based on size, reproductive tract development, and presence of thymus. Tissues were assessed for the presence or absence of gross and microscopic lesions commonly described with GI.2/RHDV2/b infection, and any additional abnormal findings were noted. Due to postmortem or euthanasia artifacts, we were not able to assess for all microscopic lesions previously reported with GI.2/RHDV2/b infection in all of our cases. In cases where artifact interfered with reliable assessment for a particular lesion, the animal was excluded from the total number assessed for that lesion.

Twelve animals were found dead, and two were found lethargic and euthanized. Five animals were submitted chilled, and nine were frozen prior to necropsy. Animals were in good to fair postmortem condition based on gross assessment. Body weights of desert cottontails ranged from 73 to 1,230 g, and weights of black-tailed jackrabbits ranged from 440 to 3,260 g. Adults were more common than juveniles (9/14 adult; 64%), and females were overrepresented (10/14 female; 71%). Of the six adult females, four were pregnant, and one was lactating. Most animals (10/14; 71%) were in poor body condition with depleted fat stores. Consistent gross findings included epistaxis (12/13; 92%; Fig. 1A); a tan friable liver with an enhanced reticular pattern or pinpoint red capsular foci (13/14; 93%; Fig. 1B); and wet and heavy lungs that were mottled pink to red or red to dark red (14/14; 100%; Fig. 1C); lungs of most (13/14; 93%) animals floated in formalin. Other common gross findings included pale tan kidneys (6/14; 43%) and meningeal congestion (5/13; 38%). Less common findings included petechiae on cecocolic serosa, epididymis, or ovary (3/14; 21%; Fig. 1D); perianal or periurethral blood (2/14; 14%); splenomegaly (2/14; 14%); and dark red tracheal mucosa (1/14; 7%). In 4/13 (31%) animals where eyes could be assessed, buphthalmia, hyphema, or hemorrhagic ocular discharge was suspected, although due to autolysis or trimming artifacts, ocular abnormalities could not be confirmed histologically.

Microscopic findings (Table 1 and Fig. 2) included dissociation (14/14; 100%) and necrosis or apoptosis (11/11; 100%) of hepatocytes; in all livers suitable for assessment of necrosis, panlobular (massive) dissociation and necrosis or apoptosis were present in at least some areas (Fig. 2C), while 3/11 (27%) livers also exhibited periportal to midzonal dissociation and necrosis or apoptosis (Fig. 2A, B). Dissociation was characterized by individualization and rounding of hepatocytes with loss of chord architecture (Fig. 2C, D). Areas of necrosis or apoptosis lacked significant heterophilic inflammation, although heterophils were present in low numbers in some areas (Fig. 2D). Less frequent hepatic findings included hemorrhage (6/9; 67%; Fig. 2B), lipid-type hepatocellular vacuolation (7/12;

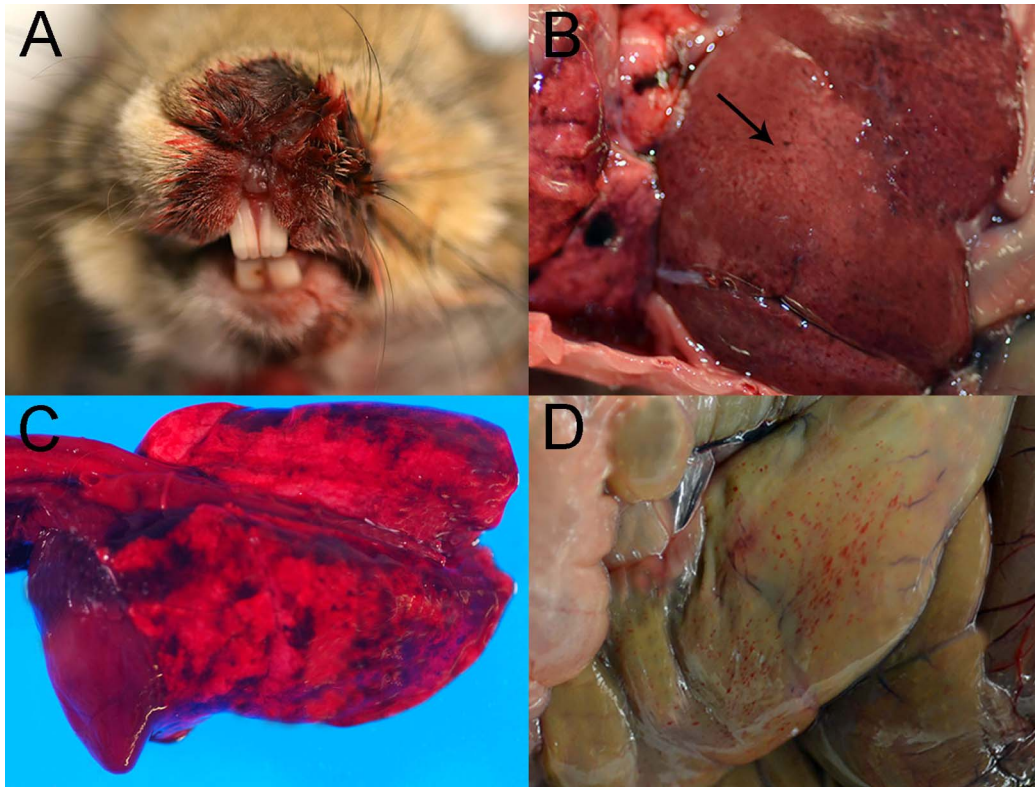

FIGURE 1. Gross lesions in *Lagovirus europaeus* GI.2/RHDV2/b (rabbit hemorrhagic disease virus 2)–positive desert cottontails (*Sylvilagus audubonii*) (A, B) and black-tailed jackrabbits (*Lepus californicus*) (C, D) from Arizona, Texas, and New Mexico, USA, collected in April and May 2020. (A) There is blood around the nose (epistaxis). (B) The liver has indistinct areas of pallor with an enhanced reticular pattern and multifocal pinpoint red capsular foci (arrow). (C) The lung contains multifocal to locally extensive dark red areas (congestion and hemorrhage). (D) There are petechial hemorrhages on the cecal serosa.

58%), and hepatocellular mineralization (4/13; 31%). Evaluation of the lung revealed congestion (12/12; 100%), edema with increased numbers of intra-alveolar macrophages (12/13; 92%), intra-alveolar hemorrhage (11/12; 92%; Fig. 2E), interstitial heterophils or mononuclear cells (2/9; 22%), and pyknotic interstitial debris (1/9; 11%). In the kidney, there was frequent accumulation of hemoglobin, red blood cells, or protein in renal tubules (9/14; 64%) and acute tubular injury (3/8; 38%; Fig. 2F). Splenic lesions were limited to congestion (2/5; 40%). Additional findings included submucosal tracheal congestion (10/13; 77%) and luminal hemorrhage (2/13; 15%); epicardial or myocardial hemorrhage (3/14; 21%); intra-alveolar fibrin accumulation (1/8; 13%); and cerebral and cerebellar hemorrhage (1/14; 7%) and meningeal congestion (1/14; 7%).

Concurrent findings not typically associated with GI.2/RHDV2/b infection and considered most probably incidental included intestinal luminal metazoan parasites or coccidia (7/14; 50%); mesenteric cysticerci (5/14; 36%); mild lymphoplasmacytic renal interstitial infiltrates (3/14; 21%); 1–2-mm-diameter white foci in the liver corresponding with granulomas seen histologically, probably secondary to *Taenia* sp. migration (2/13; 15%); subcutaneous edema (2/14; 14%); intercostal muscular hemorrhage (1/14; 7%); and eosinophilic intranuclear inclusions in the renal tubular epithelia resembling herpesviral inclusions (1/14; 7%), although a PCR for herpesvirus was negative (Ehlers et al. 1999).

TABLE 1. Microscopic findings in *Lagovirus europaeus* GI.2/RHDV2/b (rabbit hemorrhagic disease virus 2)–positive desert cottontails (*Sylvilagus audubonii*) and black-tailed jackrabbits (*Lepus californicus*) from Arizona, Texas, and New Mexico, USA, collected in April and May 2020. The number of cases assessed for each lesion varies due to tissue availability and degree of postmortem artifact.

Organ/lesion	No. animals with lesion/ no. assessed	No. desert cottontails with lesion/ no. assessed	No. black-tailed jackrabbits with lesion/ no. assessed
Liver			
Hepatocellular dissociation	14/14	7/7	7/7
Hepatocellular necrosis	11/11	5/5	6/6
Hemorrhage	6/9	4/5	2/4
Lipid-type vacuolation	7/12	2/5	5/7
Mineralization	4/13	0/6	4/7
Lung			
Congestion	12/12	6/6	6/6
Edema	12/13	7/7	5/6
Hemorrhage	11/12	6/7	5/5
Interstitial inflammation	2/9	2/6	0/3
Interstitial necrosis	1/9	0/6	1/3
Intra-alveolar fibrin	1/8	1/6	0/2
Kidney			
Renal tubular hemorrhage, hemoglobin, or protein	9/14	3/7	6/7
Acute tubular injury	3/8	1/5	2/3
Spleen			
Lymphocytolysis	0/5	0/3	0/2
Lymphoid depletion	0/4	0/3	0/1
Red pulp congestion or hemorrhage	2/5	1/2	1/3
Red pulp necrosis	0/2	0/1	0/1
Fibrin	0/4	0/2	0/2
Trachea			
Congestion	10/13	3/6	7/7
Lumenal hemorrhage	2/13	0/6	2/7
Visceral hemorrhage			
Cardiac	3/14	1/7	2/7
Brain			
Hemorrhage	1/14	0/7	1/7
Congestion	1/14	1/7	0/7

All animals tested positive for GI.2/RHDV2/b and negative for GI.1/RHDVa by real-time PCR at FADDL, and death in all cases was attributed to GI.2/RHDV2/b. Subjectively, gross lesions were more severe in the jackrabbits, and several microscopic lesions were more common in jackrabbits than in cottontails (Table 1). These included hepatocellular mineralization (57% vs. 0%); acute tubular injury (67% vs. 20%); renal tubular hemorrhage, hemoglobin, or protein (86% vs. 43%); tracheal congestion (100% vs. 50%); and tracheal luminal hemorrhage (29% vs. 0%). Hepatocellular mineralization has been previously described associated with lagoviral infection in *Lepus* spp., but not in *O. cuniculi* (Neimanis et al. 2018a). Comparing microscopic lesion prevalence in adults vs. juveniles, renal lesions were more common in adults, including renal tubular hemorrhage, hemoglobin, or protein accumulation (8/9; 89% vs. 1/5; 20%) and acute tubular injury (3/4; 75% vs. 0/4; 0%). We could not definitively determine the age of our animals, but the

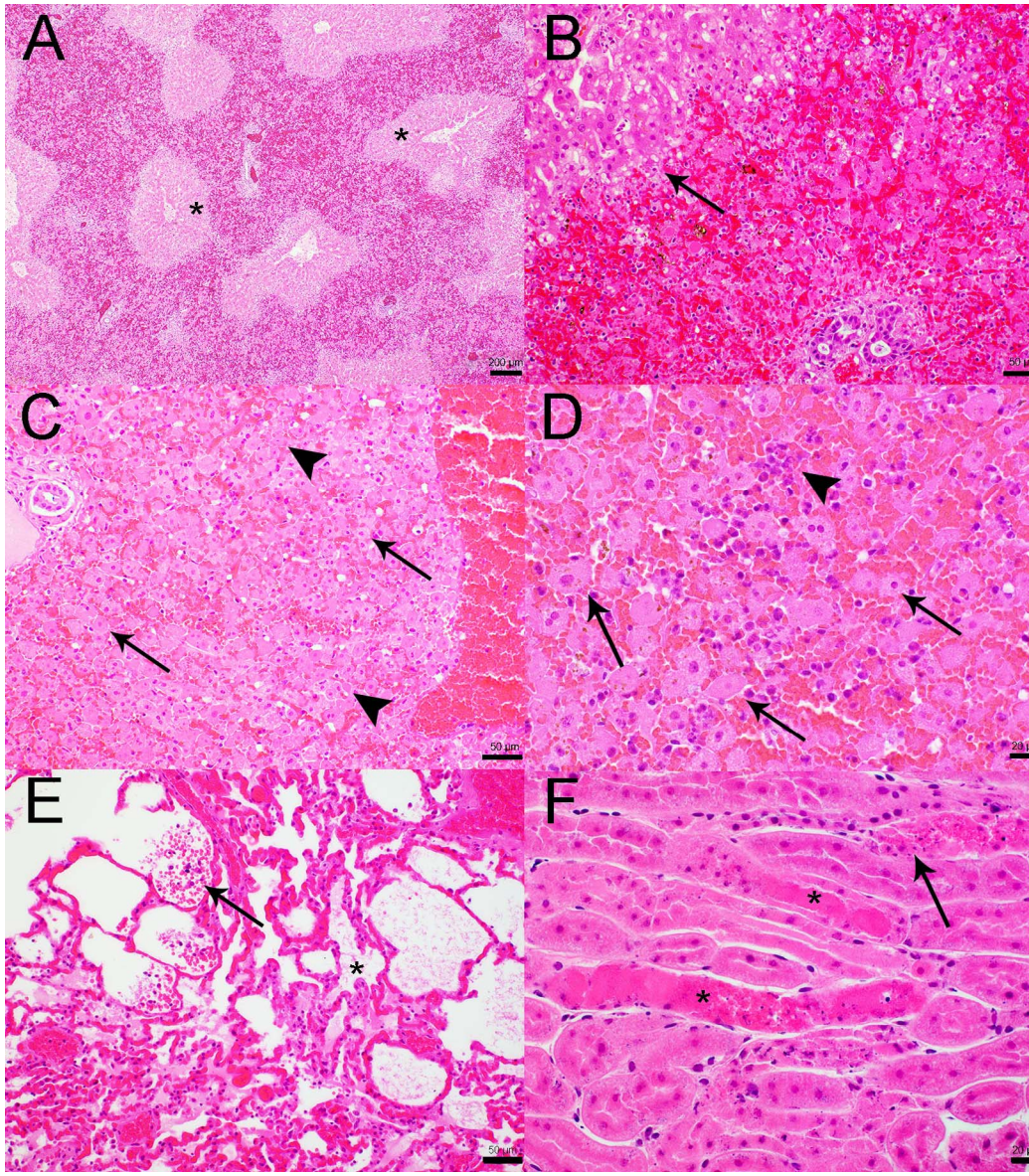

FIGURE 2. Microscopic lesions in *Lagovirus europaeus* GI.2/RHDV2/b (rabbit hemorrhagic disease virus 2)–positive desert cottontails (*Sylvilagus audubonii*) (C, D, F) and black-tailed jackrabbits (*Lepus californicus*) (A, B, E) from Arizona, Texas, and New Mexico, USA, collected in April and May 2020. H&E stain. (A) Liver. There is periportal hepatocellular dissociation and hemorrhage with loss of normal architecture. Centrilobular areas (asterisks) are relatively spared. (B) Liver. There is periportal to midzonal hepatocellular dissociation and hemorrhage. Remaining hepatocytes often exhibit discrete lipid-type vacuolation (arrow). (C) Liver. There is panlobular hepatocellular dissociation (arrows), with occasional shrinkage, hypereosinophilia, and loss of nuclear detail (necrosis or apoptosis; arrowhead), and multifocal areas of hemorrhage. (D). Liver. Dissociated hepatocytes (arrows) are occasionally admixed with low numbers of heterophils (arrowhead). (E) Lung. Alveoli multifocally contain free erythrocytes (hemorrhage; arrow) and fibrillar to homogeneous eosinophilic material (edema; asterisk). Interstitial blood vessels are congested. (F) Kidney. Renal tubules are multifocally lined by shrunken and pyknotic epithelial cells (renal tubular injury; arrow) and contain intraluminal hypereosinophilic proteinaceous material (asterisks).

inclusion of juvenile desert cottontails weighing as low as 73 g and black-tailed jackrabbits weighing as low as 440 g confirms that young animals of both species are susceptible to fatal infection, consistent with reports of fatal GI.2/RHDV2/b infection in *O. cuniculus* as young as 11 d old (Velarde et al. 2017).

Our pathologic findings associated with GI.2/RHDV2/b infection in cottontails and jackrabbits are consistent with those reported in European lagomorphs (Velarde et al. 2017; Neimanis et al. 2018a, 2018b; Harcourt-Brown et al. 2020), with few exceptions. Other lesions reported with GI.2/RHDV2/b infection in *O. cuniculus* that were not found in our cases include glomerular or pulmonary fibrin thrombi (Neimanis et al. 2018b; Harcourt-Brown et al. 2020); significant heterophilic inflammation in the liver associated with areas of necrosis (Neimanis et al. 2018b); and various splenic lesions including lymphoid depletion, lymphocytolysis, macrophage hyperplasia, red pulp necrosis, and red pulp fibrin accumulation (Neimanis et al. 2018b). It is unknown whether the absence of these findings in our animals is due to species differences, differences in the viral strain, or other factors. The number of diagnostically suitable spleens in our case set was limited due to postmortem autolysis, reducing the opportunity to detect splenic lesions.

While individualization of hepatocytes has been described previously (e.g., Neimanis et al. 2018b), the severity of hepatocellular dissociation in the livers we examined has not been emphasized in previous reports. Similarly, ocular abnormalities such as buphthalmos and blood within or around the eye have not been previously associated with GI.2/RHDV2/b infection in rabbits or hares. Although ocular abnormalities were suspected at necropsy in several animals, they could not be confirmed histologically, and their association with GI.2/RHDV2/b infection is unclear.

Based on the microscopic findings, death was probably acute to subacute; however, most rabbits and hares in this report were in poor body condition. It is unknown whether animals in poor body condition may have been more likely to become infected with GI.2/RHDV2/b or to have developed lethal disease, or whether poor body condition was an incidental finding. In a previous report, body condition of wild lagomorphs with GI.2/RHDV2/b infection varied from normal to emaciated (Neimanis et al. 2018a).

Compared to desert cottontails (n=8) and black-tailed jackrabbits (n=7) in the National Wildlife Health Center diagnostic database that were examined prior to 2020 or tested PCR-negative for GI.2/RHDV2/b at FADDL, the presence of massive hepatocellular dissociation with necrosis or apoptosis was the most diagnostically distinct feature of GI.2/RHDV2/b infection, as both epistaxis (11/15; 73%) and pulmonary edema or congestion (11/15; 73%) were common postmortem findings in GI.2/RHDV2/b–negative animals or those predating the introduction of GI.2/RHDV2/b into the North American wild lagomorph population, while hepatocellular dissociation was not noted in any case. The relative specificity of hepatocellular necrosis or apoptosis for GI.2/RHDV2/b infection was also posited by Harcourt-Brown et al. (2020) for pet *O. cuniculus*.

In Europe, wild rabbit population declines and associated ecologic changes, including declines in endangered predator populations, have been attributed to the introduction of GI.2/RHDV2/b (Monterroso et al. 2016). Given the susceptibility of both *Sylvilagus* spp. and *Lepus* spp., further efforts are needed to understand, control, and mitigate the impacts of GI.2/RHDV2/b in native North American lagomorphs.

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